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Three new species of blood flukes (Digenea: Aporocotylidae) infecting pufferfishes (Teleostei: Tetraodontidae) from off Bali, Indonesia

R.Q-Y. Yong[1*], S.C. Cutmore[1], R.A. Bray[2], T.L. Miller[3, 4], I.W.Y. Semarariana[5], H.W. Palm[5, 6] & T.H. Cribb[1]

[1] School of Biological Sciences, The University of Queensland, Brisbane, Queensland 4072, Australia

[2] Department of Life Sciences, Natural History Museum, Cromwell Road, London SW7 5BD, United Kingdom

[3] Fish Health Laboratory, Department of Fisheries Western Australia, 3 Baron-Hay Court, South Perth, Western Australia 6151, Australia

[4] Centre for Sustainable Tropical Fisheries and Aquaculture, College of Marine and Environmental Sciences, James Cook University, Cairns, Queensland 4878, Australia

[5] The Faculty of Veterinary Medicine, Sudirman Campus, Universitas Udayana, Jalan Kampus Udayana, Denpasar, 80361, Indonesia

[6] Faculty of Agricultural and Environmental Sciences, Aquaculture and Sea-ranching, University Rostock, Justus-von-Liebig Weg 6, 18059, Rostock, Germany

* Corresponding author: The University of Queensland, School of Biological Sciences, Brisbane, Queensland 4072, Australia. Tel: +61 7336 3294. E-mail: rqy.yong@uqconnect.edu.au

Abstract

We describe three new species of blood flukes (Aporocotylidae) and propose their classification within the genus *Psettarium* Goto & Ozaki, 1929. All three species were collected from the circulatory systems of pufferfishes caught off Bali, central Indonesia. *Psettarium pulchellum* n. sp. was found in the gills of both the narrow-lined puffer (*Arothron manilensis* de Procé) and the spiny blaasop (*Tylerius spinosissimus* Regan), while *P. ogawai* n. sp. and *P. jimbaranensis* n. sp. were found in the gills of the reticulated puffer (*Arothron reticularis* Bloch & Schneider). The morphological characteristics of these taxa necessitated emendation of the diagnosis for the genus *Psettarium*, to accommodate the presence of an oral sucker, multiple or entirely post-caecal testes and a degenerate posterior testis. Features such as proportion of body length occupied by the oesophagus, and posterior caeca being ≥7× the length of anterior caeca, are no longer regarded as useful genus-level characters. Additionally, *Sasala nolani* is reassigned to this genus as *Psettarium nolani* n. comb. In phylogenetic analyses of the 28S and ITS2 rDNA regions, all three new taxa form a well-supported clade, together with *Psettarium sinense* and *Psettarium nolani* n. comb., the two other species of tetraodontid-infecting aporocotylids for which comparative rDNA data were available. The short branch lengths within this clade, despite dramatic morphological differences between the five species, suggest that rapid morphological diversification has occurred among the tetraodontid-infecting aporocotylids. The genus *Psettarium* has long been considered problematic. Further commentary is given on the history of this genus and how the issues presented might be resolved.

Key words: *Psettarium*, blood flukes, speciation, Coral Triangle, degenerate posterior testes

1. Introduction

Central Indonesia is located in a hotspot of global marine biodiversity, being in the Coral Triangle region bound by the Philippines in the north, Peninsular Malaysia in the west and Solomon Islands in the east [1, 2]. The region's importance as a centre for diversification and endemism means that the fish fauna is particularly well-studied, although new species are still being described (e.g. [3-5]). By contrast, the parasitic fauna of Indonesian fishes is comparatively poorly studied. Dedicated taxonomic studies have been scarce, with recent focus being on species of commercial aquaculture significance such as epinepheline serranids and the Asian sea-bass *Lates calcarifer* Bloch (e.g. [6-8]). The only systematic examination of the digenean fauna of Indonesian fishes was performed in the early 1950s by S. Yamaguti, who described 58 of the roughly 80 digenean species known from Indonesian fishes, primarily from the Sulawesi region [9, 10]. The new species described in this study form part of a wider parasitological collection made between July and August 2013, in coastal marine waters off Bali, east of Java, as part of 'The First Educational Workshop on Marine Fish Parasites in Indonesia' held in Denpasar, Bali. This is the first time that the fish fauna of this area has been systematically surveyed for parasites.

The aporocotylid blood flukes are digeneans that infect the circulatory systems and body cavities of fishes; members of the family are found especially in the heart and gills, but also the cranial and mesenteric blood vessels of a wide range of fishes (see [11] for a review of aporocotylid infection sites). Reports of aporocotylids have historically lagged behind those of other trematodes [12], but the family has recently received increasing attention, first because of increases in taxonomic studies, and second due to certain species' significance to fisheries and aquaculture as agents of host disease and mortality (e.g. [13-16]). These investigations have resulted in both descriptions of new species and the elucidation of complete life cycles [17, 18]. New aporocotylid species are now frequently being described from a diverse range of fishes, both due to the increased attention paid in fisheries or aquaculture studies, as well as more general investigations into richness of the family [19-23].

Known aporocotylid hosts include the highly-derived teleost order Tetraodontiformes, primarily from the pufferfish family Tetraodontidae. To date, seven aporocotylid species have been described from the Tetraodontidae. Here, we describe three new species of aporocotylid from two species of pufferfish of the genus *Arothron* Müller. We also report one of the new species from the spiny blaasop, *Tylerius spinosissimus* Regan. The only previous record of aporocotylid flukes from Indonesia is a single record of an unidentified species

from maricultured sea-bass (*L. calcarifer*) from Lampung Bay, Sumatra [6]. These are thus the first aporocotylid flukes to be described from Indonesian waters.

2. Materials and Methods

2.1 Sample collection

Pufferfishes were obtained from the vicinity of Kedonganan Beach, Bali, Indonesia (8°45'S, 115°10'E), from July-August 2013. All fishes were collected by local fishermen from the surrounding Jimbaran Bay area using fish traps. The heart, branchial arteries and gills were examined for worms, while squashes of gill and heart tissue were examined for the presence of eggs following Yong *et al*. [24]. Aporocotylids were heat-fixed in near-boiling saline and stored in 70% ethanol.

2.2 Morphological analysis

Specimens intended for morphological analysis were washed in fresh water and stained with Mayer's haematoxylin. Specimens were then de-stained with 1% HCl, neutralised in 1% ammonia solution and dehydrated in a graded series of ethanols. Specimens were then cleared in methyl salicylate and mounted in Canada balsam. Drawings were made with the aid of a camera lucida mounted on an Olympus BH-2 light microscope, and digitised using Adobe Illustrator 6.0 (Adobe). Measurements were made using a SPOT Insight™ digital camera (Diagnostic Instruments, Inc.) mounted on an Olympus BH-2 light microscope using SPOT™ imaging software. All measurements are in micrometres (µm) and given as a range with the mean in parentheses. Where breadth follows length, the two measurements are separated by '×'. Type-specimens are deposited in the National Biodiversity Collection of the Zoological Museum of Bogor, Java, Indonesia (ZMB), the Natural History Museum, Berlin, Germany (Generalkatalog Entozoa) (NHMB), and the Queensland Museum, Brisbane, Australia (QM).

2.3 Molecular sequencing

Specimens sequenced during this study are listed in Table 1. Total genomic DNA was extracted using standard phenol/chloroform extraction techniques [25]. Partial 28S nuclear ribosomal DNA was amplified using the primers LSU5 (5'-TAG GTC GAC CCG CTG AAY

TTA AGC-3') [26] and either 1200R (5'-GCA TAG TTC ACC ATC TTT CGG-3') [26] or 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′) [27] and the ITS2 region using the primers 3S (5'-GGT ACC GGT GGA TCA CGT GGC TAG TG-3') [28] and ITS2.2 (5'-CCT GGT TAG TTT CTT TTC CTC CGC-3') [29]. PCR for both 28S and ITS2 regions was performed with a total volume of 20 µl consisting of approximately 10 ng of DNA, 5 µl of 5 x MyTaq Reaction Buffer (Bioline), 0.75 µl of each primer (10 pmols) and 0.25 µl of Taq DNA polymerase (Bioline MyTaq™ DNA Polymerase), made up to 20 µl with Invitrogen™ ultraPURE™ distilled water. Amplification was carried out on a MJ Research PTC-150 thermocycler. Amplification of the 28S region was carried out using the following profile: an initial denaturation for 4 min at 95°C, followed by 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 56°C, extension for 2 min at 72°C, followed by a single cycle of denaturation for 1 min at 95°C, annealing for 45 sec at 55°C and a final extension for 4 min at 72°C. Amplification of the ITS2 region was carried out using the following profile: an initial single cycle of denaturation for 3 min at 95°C, annealing for 2 min at 45°C, extension for 90 sec at 72°C, followed by 4 cycles of denaturation at 95°C for 45 sec, annealing for 45 sec at 50°C, extension for 90 sec at 72°C, followed by 30 cycles of denaturation for 20 sec at 95°C, annealing for 20 sec at 52°C, extension for 90 sec at 72°C, and a final extension for 5 min at 72°C. Amplified DNA was purified using a Bioline ISOLATE II PCR and Gel Kit, according to the manufacturer's protocol. Cycle sequencing of purified DNA was carried out using the same primers used for PCR amplification as well as the additional 28S primers 300F (5'-CAA GTA CCG TGA GGG AAA GTT-3') [26] and ECD2 (5′-CTT GGT CCG TGT TTC AAG ACG GG-3′) [26], and the additional ITS2 primer GA1 (5'-AGA ACA TCG ACA TCT TGA AC-3') [30]. Cycle sequencing was carried out at the Australian Genome Research Facility using an AB3730xl capillary sequencer. Sequencher™ version 4.5 (GeneCodes Corp.) was used to assemble and edit contiguous sequences.

2.4 Phylogenetic analysis

The ITS2 and partial 28S rDNA sequences generated in this study (Table 1) were aligned with sequence data of other species of aporocotylids that were available on GenBank (Table 2). Several sequences published on GenBank were excluded from the final analyses due to their short length (Table 2). Alignments were performed using MUSCLE 3.7 [31] with ClustalW sequence weighting and UPGMA clustering for iterations 1 and 2. The resultant alignments were refined by eye using MESQUITE [32], with the ends trimmed to match the shortest sequence in the alignments. Bayesian inference and Maximum Likelihood analyses of the partial 28S rDNA dataset were conducted to explore relationships between these taxa.

Bayesian inference analysis was conducted using MrBayes v3.2.2 [33], run on the CIPRES portal [34]. Maximum Likelihood analysis was conducted using RAxML v7.2.8 [35]. jModelTest v0.1.1 [36] was used to estimate the best nucleotide substitution model for the dataset. Bayesian inference analysis was conducted using the TVM+I+G model, predicted as the best estimator by the Akaike Information Criterion (AIC) in jModelTest. The closest approximation of this model was implemented in the subsequent Maximum Likelihood analysis. Bayesian inference analysis was run over 10,000,000 generations (ngen = 10000000) with two runs each containing four simultaneous Markov Chain Monte Carlo (MCMC) chains (nchains = 4) and every 1,000th tree saved (samplefreq = 1000). Bayesian inference analysis used the following parameters: nst = 6, rates = invgamma, ngammacat = 4, and the priors parameters of the combined dataset were set to ratepr = variable. Samples of substitution model parameters, and tree and branch lengths were summarised using the parameters 'sump burnin = 3000' and 'sumt burnin = 3000'. Tracer software [37] was used to visually assess when log likelihood values stabilized for setting burnin values in the Bayesian analyses. These 'burnin' parameters were chosen because the log likelihood scores 'stabilised' well before 3,000,000 replicates. Nodal support in the Maximum Likelihood analysis was estimated by performing 100 bootstrap pseudoreplicates. The ITS2 rDNA dataset was analysed *via* Maximum Likelihood analysis using the GTR+I+G model, predicted as the best estimator by the Akaike Information Criterion (AIC) in jModelTest, performed using MEGA 6 [38] with the 'use all sites' parameter selected. Species of Spirorchiidae were designated as functional outgroups in all analyses. Total and uncorrected 'p' pairwise nucleotides distances for taxa in the 28S and ITS2 datasets were calculated using MESQUITE with gaps treated as a character state.

3. Results

3.1 Psettarium Goto & Ozaki, 1930 (modified after Bullard & Overstreet, 2006)

Diagnosis: Body slender or broad, lanceolate, flattened, ventrally concave, possessing sinistro-lateral protuberance at level of genital pores. Tegumental body spines in marginal transverse rows, minute, not curved or hooked. Spine rows mostly continuous along length of body, occasionally discontinuous at regions adjacent to excretory pore. Rosethorn-shaped or fused spines absent. Oral sucker present or absent. Mouth ventrally subterminal. Pharynx absent. Oesophagus weakly sinuous, to 40% of body length. Caeca X-shaped; anterior caeca equal to subequal in length, not sinuous, with varying lateral diverticulation; posterior caeca longer than anterior caeca, subequal to greatly unequal in length, sinuous, sometimes possessing lateral diverticulation or thornlike projections. Anterior testes 1 to 22, massive or

compact, intercaecal or post-caecal, with margins that may or may not extend laterally beyond caeca or lateral nerves, smooth or deeply lobed. Degenerate posterior testis present or absent; if present, posterior to rest of reproductive system, close to posterior extremity of body. Cirrus-sac sinistral, posterior to primary gonads but anterior to degenerate posterior testis. Male genital pore marginal to submarginal, sometimes at tip of sinistro-lateral protuberance. Ovary post-caecal, post-testicular, medial or dextral in body, with margins deeply lobed or smooth. Oviduct arises from posterior margin of ovary, passes posteriorly to oötype forming poorly-delineated oviducal seminal receptacle, then anteriorly becoming uterus. Oötype distinct, posterior to rest of terminal genitalia, except when degenerate posterior testis present. Uterine coils irregularly convoluted, wholly sinistral to oviduct, occupying space posterior to ovary and anterior to oötype, with final coils extending anteriorly as far as posterior margin of ovary, then descending posteriorly to open at genital pore. Metraterm indistinct. Female genital pore dorso-sinistral, anterior and dextral to male pore. Vitelline follicles variably distributed, from anterior extremity to level of testis, ovary or genital pores. Parasitic in heart, gills, cardiac and mesenteric vessels of marine teleosts of the families Mullidae, Rachycentridae and Tetraodontidae. Type-species *Psettarium japonicum* (Goto & Ozaki, 1929) Goto & Ozaki, 1930.

3.2 Psettarium jimbaranensis n. sp.

Description (Fig. 1A, B; 2A): [Based on 2 whole-mounted specimens, including 1 hologenophore (anterior end only)]. Body lanceolate, 7.6 × longer than maximum breadth, ventrally concave, margins narrow slightly unequally at level of male genital pore, 1460 × 192. Tegumental spines minute, straight, less than 2 long. Spines arranged in marginal transverse rows that wrap dorsoventrally; rows begin just posterior to oral sucker, initially 6 in length and spaced 2 apart, bearing 4–5 spines each, increasing within first 10 rows to 8–9 in length and spaced 4 apart, bearing 8–9 spines each, before decreasing towards posterior extremity to 6 in length and spaced 2 apart, with rows ending 12 from posterior extremity. Nerve cords 7 in diameter, run length of body 45 from body margins, well-defined. Dorsal nerve commissure 99 from anterior extremity, 35 across. Oral sucker well defined, aspinous, 23–29 × 35–44 (26 × 40). Mouth a simple ventrally subterminal pore, 9 from anterior extremity. Oesophagus 441–609 (525), 30.2% of total body length. Intestinal bifurcation medial in anterior half of body. Anterior caeca straight with minimal diverticulation, subequal in length. Left anterior caecum 112–144 (128); right anterior caecum 114–126 (120). Posterior caeca sinuous, unequal in length. Left posterior caecum 430–535 (483); right

posterior caecum 372–388 (380). Posterior caeca 3.0–4.7 (3.8) × longer than anterior caeca; total caecal length 44.5% of total body length.

Anterior testis single, massive, unlobed, margins irregular, inter-caecal, with margins extending laterally beyond caecal margins, almost to nerve cords, posteriorly well beyond extent of posterior caeca, 655 × 111. Vas deferens originates medially from posterior margin of testis, passes under uterine bend before forming primary seminal vesicle. Seminal vesicle turgid in available specimen, well-defined, bifurcates above posterior margin of cirrus-sac, with one duct leading to cirrus-sac, and other duct passing dextrally dorsal to uterus and ventral to oviduct and vitelline duct, meeting degenerate posterior testis. Degenerate osterior testis well-defined, medial, posterior to rest of genitalia, 38 from posterior extremity and 22 from dextral lateral margin of body, 46 × 59, containing undifferentiated cells and large volume of sperm. Cirrus-sac reniform, filled mainly by mass of glandular cells surrounding broad, poorly-delineated duct, 74 × 36. Male genital pore dorso-sinistral, elevated from body on indistinct dorsally-directed protuberance, opening 22 from body margin.

Ovary oblong, margins lobed, immediately posterior to testis, 35–49 × 68–106 (44 × 84). Oviduct originates medially from posterior margin of ovary, passes posteriorly, then anteriorly to meet oötype. Uterus irregularly coiled, passing anteriorly almost to posterior ovarian margin, then posteriorly to open at female genital pore. Female genital pore dorso-sinistral, anterior and dextral to cirrus-sac and male genital pore; 38–63 (49) from body margin, 161–203 (179) from posterior extremity. Vitelline follicles extensive, dense, evenly distributed from near anterior extremity to level of female genital pore, extending laterally beyond nerve cords almost to body margins. Vitelline duct runs medially, traceable from near caecal bifurcation to oötype, dorsal to rest of genitalia.

Excretory system indistinct. Excretory pore terminal.

3.3 Taxonomic summary

Type-host: *Arothron reticularis* Bloch & Schneider, reticulated pufferfish (Tetraodontidae).

Type-locality: Kedonganan Beach (8°45'S, 115°10'E), Jimbaran, southern Bali, Indonesia.

Site: Gill arches and gill filaments.

Prevalence: 1 of 1 fish infected with 2 worms.

Type-material: ZMB: xxxxxxxx; NHMB: xxxxxx.

Molecular sequence data: See Table 1.

Etymology: The species name refers to the Jimbaran area of southern Bali, where the host fish was obtained.

3.3 Psettarium ogawai n. sp.

Description (Fig. 1C, D; 2B; 3): [Based on 14 whole-mounted specimens, including one hologenophore (posterior end only)]. Body narrowly lanceolate, 10.2–14.7 (12.0) × longer than maximum breadth, with margins narrowing at level of female genital pore, then broadening, creating paddle-like shape to posterior end, more pronounced on sinistral side; body dorsally convex, with distinct dorsal 'hump' created by cirrus-sac, 1254–1939 × 99–167 (1511 × 126). Tegumental spines minute, straight, less than 1 long. Spines arranged in marginal transverse rows that wrap dorsoventrally; first row 7–8 from base of oral sucker, last rows 5 from excretory pore. Row length initially 3, bearing 5–6 spines each, increasing within 4 rows to 7, bearing 9–11 spines, decreasing in length to 3 in last 4–5 rows towards posterior extremity, with spines decreasing to 5–6 per row. Nerve cords well-defined, 4–6 (5) in diameter, run length of body 16–27 (22) from body margins. Dorsal nerve commissure 63–100 (77) from anterior extremity, 25–42 (33) across. Oral sucker well defined, with minute paired terminal papillae (Fig. 3), 13–22 × 21–33 (17 × 26). Mouth a simple pore, terminal to just ventrally subterminal. Oesophagus weakly sinuous, sinuations becoming more pronounced posteriorly, 388–602 (466), 28.9–34.1% (30.8%) of body length. Intestinal bifurcation medial in anterior half of body. Anterior caeca straight, with minimal diverticulation, equal to subequal in length. Left anterior caecum 68–169 (113); right anterior caecum 81–134 (102). Posterior caeca straight to sinuous, with irregular short diverticula and posterior extremities often swollen, equal to subequal in length, run tightly parallel to one another. Left posterior caecum 409–588 (478); right posterior caecum 339–696 (458). Posterior caeca 3.5–5.7 (4.4) × longer than anterior caeca; total caecal length occupying 37.5–44.6% (41.0%) of body length.

Anterior testes 16–22 (20), arranged in diagonally alternating pairs; anterior-most testis occupying space next to posterior extremity of longer posterior caecum. Individual testes spherical or nearly so, unlobed, 19–37 × 24–36 (28 × 30). Testicular field does not exceed lateral nerve cords; total area 226–386 × 62–97 (293 × 73). Vas deferens originates medially from posterior margin of testicular field, passes under uterine coils and bifurcates just posterior to cirrus-sac, one duct leading to cirrus-sac and other to degenerate posterior testis. Degenerate osterior testis spherical or nearly so, varying in turgidity and sperm

content, medial, just posterior to remainder of genitalia, 35–59 (45) from posterior extremity, 20–40 × 19–30 (27 × 23). Cirrus-sac reniform, mainly filled by mass of glandular cells surrounding broad, indistinct duct, 59–101 × 26–49 (71 × 35). Male genital pore dorso-sinistral, marginal or nearly so.

Ovary spherical or nearly so, unlobed, on dextral side of body, mostly post-testicular, 38–67 × 33–57 (50 × 41). Oviduct originates dextrally from posterior margin of ovary, passes medially and posteriorly, then anteriorly to meet oötype. Uterus irregularly coiled, passing anteriorly almost to posterior ovarian margin, then posteriorly to meet female genital pore. Female genital pore dorso-sinistral, anterior and dextral to cirrus-sac and male genital pore, 19–35 (25) from body margin, 147–303 (201) from posterior extremity. Vitelline follicles in large bundles arranged in mosaic pattern, entirely confined by nerve cords, extend from dorsal nerve commissure to level of ovary, concentrated around and between oesophagus and caeca, becoming sporadic at level of gonads. Vitelline duct runs medially from near caecal bifurcation to oötype, dorsal to remainder of genitalia.

Excretory system indistinct. Excretory pore terminal.

3.4 Taxonomic summary

Type-host: *Arothron reticularis* Bloch & Schneider, reticulated pufferfish (Tetraodontidae).

Type-locality: Kedonganan Beach (8°45'S, 115°10'E), Jimbaran, southern Bali, Indonesia.

Site: In gill arches and gill filaments.

Prevalence: 1 of 1 fish infected with 28 worms.

Type-material: ZMB: xxxxxxxx–xxxxxxxx; QM: xxxxxx–xxxxxx; NHMB: xxxxx–xxxxx.

Molecular sequence data: See Table 1.

Etymology: The species is named for Prof Kazuo Ogawa, Director of the Meguro Parasitological Museum, Japan, in recognition of his extensive work on aporocotylid taxonomy and biology.

3.5 Psettarium pulchellum n. sp.

Description (Fig. 1E; 2C): [Based on 14 whole-mounted specimens, including 2 hologenophores (posterior end only)]. Body broadly lanceolate, 4.3–5.7 (5.2) × longer than maximum breadth, broadest at midsection, ventrally concave, with margins nearly equal, except for slight narrowing on sinistral margin at level of male genital pore, creating asymmetrical posterior 'bend', 1395–1703 × 261–338 (1537 × 297). Tegumental spines minute, straight, less than 2 long, arranged in marginal transverse rows that wrap dorsoventrally; rows begin 36–62 (50) from anterior extremity, or 15–26 (19) from base of oral sucker, initially 7 in length and spaced 2 apart, bearing 4–5 spines each dorsally as well as ventrally, increasing within first 10 rows to 15–16 in length and spaced 4 apart, bearing 7–8 spines each dorsally as well as ventrally, before decreasing towards posterior extremity to 6 in length and spaced 2 apart, with rows ending 12–14 from posterior extremity. Nerve cords 7–10 (8) in diameter, well defined, run length of body 46–71 (60) from body margins. Dorsal nerve commissure 107–131 (122) from anterior extremity, 49–72 (60) across. Oral sucker well defined, aspinous, 23–30 × 35–56 (26 × 45). Mouth ventrally subterminal, 9–15 (11) from anterior extremity, a simple pore to 5 in diameter. Oesophagus weakly sinuous, with sinuations becoming more pronounced posteriorly, 464–658 (571), occupying 33.3–39.5 (37.2)% of body length. Intestinal bifurcation medial in anterior half of body. Anterior caeca straight with minimal diverticulation, equal to subequal in length. Left anterior caecum 104–193 (153); right anterior caecum 117–215 (159). Posterior caeca sinuous, with short irregular diverticula, equal to subequal in length. Left posterior caecum 405–603 (530); right posterior caecum 318–629 (494). Posterior caeca 2.7–4.0 (3.2) × longer than anterior caeca; total caecal length occupying 40.5–49.6 (45.1)% of total body length.

Anterior testis single, spherical or nearly so, unlobed, entirely post-caecal, 90–150 × 84–136 (114 × 120). Vas deferens originates medially from posterior margin of anterior testis, passes posteriorly forming seminal vesicle. Seminal vesicle poorly defined, bifurcates just anterior to anterior margin of cirrus-sac, with one duct leading to cirrus-sac and second duct leading to degenerate posterior testis. Degenerate posterior testis a delicate structure varying in turgidity and sperm content, posterior to remainder of genitalia, 29–63 (44) from posterior extremity and 18–41 (27) from sinistral lateral margin of body, 28–50 × 9–23 (39 × 18). Cirrus-sac reniform, mainly filled by mass of glandular cells surrounding broad, poorly-delineated duct, with anterior portion forming protrusible papilla 7 long, 67–102 × 28–41 (82 × 34). Male genital pore dorso-sinistral, opens 15 from body margin.

Ovary a smooth oblong to oblate spheroid, immediately posterior to testis, 35–49 × 68–106 (44 × 84). Oviduct originates medially from posterior margin of ovary, passes posteriorly, then anteriorly to meet oötype. Uterus irregularly coiled, passing anteriorly almost to posterior margin of ovary, then posteriorly to open at female genital pore. Female genital

pore dorso-sinistral, anterior and dextral to cirrus-sac and male genital pore, 38–63 (49) from body margin, 161–203 (179) from posterior extremity. Vitelline follicles almost entirely confined by nerve cords, extend from dorsal nerve commissure to ovary, mostly concentrated around and between posterior caeca. Vitelline duct medial, traceable from near caecal bifurcation to oötype, dorsal to remainder of genitalia.

Excretory vesicle seen in one specimen, medial, 19 from posterior extremity, 15 × 8; collecting ducts indistinct. Excretory pore terminal.

3.6 Taxonomic summary

Type-host: *Arothron manilensis* de Procé, narrow-lined pufferfish (Tetraodontidae).

Other host: *Tylerius spinosissimus* Regan, spiny blaasop (Tetraodontidae).

Type-locality: Kedonganan Beach (8°45'S, 115°10'E), Jimbaran, southern Bali, Indonesia.

Site: *A. manilensis*: Adult worms in gills. Trapped eggs seen in both heart and gills; *T. spinosissimus*: Unknown. Adult worms found in body wash.

Prevalence: *A. manilensis*: 1 of 2 fish infected with 15 worms; *T. spinosissimus*: 1 of 2 fish infected with 2 worms.

Type-material: ZMB: xxxxxxxx–xxxxxxxx; QM: xxxxxx–xxxxxx; NHMB: xxxxx–xxxxx.

Molecular sequence data: See Table 1.

Etymology: The species name *pulchellum* is derived from the diminutive of the Latin word *pulcher*, meaning ‘beautiful’, in reference to the neat, elegant organisation of this worm’s anatomy.

3.7 Molecular results

Alignment of the partial 28S rDNA dataset yielded 1,208 characters for analysis. Several published sequences were excluded from the final analyses due to their short length (see Table 2 for list of excluded sequences); inclusion of these sequences in preliminary analyses (not shown) did not change the overall topology of the phylograms produced. Bayesian inference and Maximum Likelihood analyses produced phylograms with identical topologies (Fig. 5). In both analyses, the three new taxa, along with two others that infect tetraodontids,

Sasala nolani Bray, Cribb & Littlewood, 2012 and *Psettarium sinense* (Liu, 1997), formed a strongly-supported clade. This clade of tetraodontid-infecting taxa showed short branch lengths between its member species, and was sister to *Skoulekia meningialis* Alama-Bermejo, Montero, Raga & Holzer, 2011 from the Mediterranean sparid *Diplodus vulgaris* Geoffroy Saint-Hilaire. The *Paradeontacylix* McIntosh, 1934, clade is notable for showing similarly short branch lengths between species.

Alignment of the ITS2 rDNA dataset yielded 615 characters for analysis. Maximum Likelihood analysis of the ITS2 dataset resulted in a phylogram with topology consistent with that of the 28S analyses, in that taxa infecting tetraodontids form a well-supported clade sister to *Skoulekia meningialis*. Nodal support for many of the clades was lower than in the 28S analyses, thus the phylogram is not presented here. The pairwise nucleotide differences between the species of tetraodontid-infecting species, for both the 28S and ITS2 regions, are provided in Table 3.

Our molecular analysis showed a single base difference in the ITS2 region between a specimen of *P. pulchellum* n. sp. found in *Tylerius spinosissimus* and three replicates from *Arothron manilensis*. Similarly, a single base-pair difference in the ITS2 region was observed between replicates of *P. ogawai* n. sp. The single nucleotide difference observed in each of these species are located at non-homologous positions in the ITS2 and were both transitions. In both cases, however, no variation was seen in the 28S region. We thus interpret these single-base differences as intra-specific variation.

4. Discussion

4.1 Overview

Analysis of both partial 28S and ITS2 rDNA data for the three new species and two others that infect tetraodontid fishes (*Psettarium sinense* and *Sasala nolani*) indicates that these five taxa form a strongly supported clade to the exclusion of all other aporocotylids for which molecular data are available. This clade includes four of the six aporocotylid species reported from species of *Arothron*; no sequence data are available for *Paracardicola hawaiensis* Martin, 1960 or *Rhaphidotrema kiatkiongi* Yong & Cribb, 2011. Despite the substantial morphological variation encompassed by the species of this clade, we conclude that all are best regarded as species of *Psettarium*. This conclusion means that the genus *Psettarium* requires rediagnosis, including changes with regard to the proportion of body length occupied by the oesophagus and the relative length of the anterior and posterior

caeca, as well as the presence of an oral sucker and a degenerate posterior testis. Additionally, we no longer regard testis morphology as being a usefully diagnostic feature of this genus. Our justifications for these proposed changes are detailed below. The rediagnosis of *Psettarium* means there are insufficient grounds to maintain *Sasala nolani* in a separate genus. We thus propose the recombination of *S. nolani* with *Psettarium*, as *Psettarium nolani* (Bray, Cribb & Littlewood, 2012) n. comb. *Psettarium* has a complex taxonomic history and many aspects of its recognition remain unresolved. Below we review these issues in the light of our new data.

4.2 The genus Psettarium

The genus *Psettarium* was erected by Goto & Ozaki [39] as a replacement name for *Plehnia* Goto & Ozaki, 1929, which was preoccupied. *Psettarium japonicum* (Goto & Ozaki, 1929), the type-species for this genus, was originally described as infecting the intestine of the pufferfishes *Takifugu pardalis* Temminck & Schlegel and *T. rubripes* Temminck & Schlegel. We regard the description of this infection site as likely to be, at best, misleading; bearing in mind that several species of clearly-related aporocotylids infect the mesenteric vessels of their hosts (including most of the tetraodontid-infecting species), it seems highly probable that the specimens were from that site instead. Goto & Ozaki [40] (Figs. 13 & 14, pp. 378–379) had difficulty distinguishing the testis of *P. japonicum*, with the illustration seemingly not differentiating it from the vitelline follicles; they did, however, describe the testis in single rather than plural terms. This observation was borne out by Yamaguti's redescription of *P. japonicum* [41]. Yamaguti [42], however, then altered the diagnosis of *Psettarium* to incorporate multiple testes. As far as can be determined, the type-specimens of this species have been lost. Redescription and molecular characterisation of this species is highly desirable for the refinement of the understanding of the genus *Psettarium*.

Manter [43] described *Psettarium tropicum* Manter, 1940 from the tetraodontid *Sphoeroides annulatus* (Jenyns) from off Ecuador. The infection site was not ascertained, but postulated to be the mesenteric vessels, with the worms being obtained from "washings of the coelom and once from the intestine" [43]. Manter was uncertain about the number of testes present, stating they were "diffuse, indistinct, with boundaries too indefinite to allow counting...almost indistinguishable from vitellaria". Yamaguti [42] regarded *P. tropicum* as having a single large testis, and in rediagnosing *Psettarium*, *P. tropicum* was transferred to *Cardicola* Short, 1953 (as *Cardicola tropicus*).

Lebedev & Parukhin [44] reassigned *C. tropicus* to the new genus *Psettarioides* Lebedev & Parukhin, 1972, along with several other species: *P. pseudupenei* Lebedev & Parukhin, 1972, from the mullid *Parupeneus indicus* (Shaw), *P. rachycentri* Lebedev & Parukhin, 1972 from the rachycentrid *Rachycentron canadum* Linnaeus, and *Cardicola grandis* Lebedev & Mamaev, 1968 from marlins and sailfish (Istiophoridae); *Psettarioides kurochkini* Lebedev & Parukhin, 1976, from the mullid *Mullus barbatus* Lacepède, was added later [45]. Bullard & Overstreet [46] argued that Lebedev & Parukhin [44] provided inadequate diagnosis of *Psettarioides*, and returned *P. tropicum* to *Psettarium*, transferred *P. pseudupenei* (tentatively) and *P. rachycentri* to *Psettarium*, *P. kurochkini* to *Cardicola* and *P. grandis* assigned as *incertae sedis*, acknowledging that in most of these cases, the lack of detail in the descriptions and inability to examine specimens meant that their reassignments were tentative. Bullard & Overstreet [46] also described an additional species of *Psettarium*, *P. anthicum* Bullard & Overstreet, 2006, from *R. canadum*, the second species of *Psettarium* from this fish. Most recently, on the basis of repeated molecular phylogenetic analysis [14, 20, 47-49], a third species from the body cavity of a tetraodontid was transferred to *Psettarium* as *P. sinense* (Liu, 1997) [49]. Thus, as most recently reviewed, *Psettarium* comprises six species, of which three, including the type-species, infect (probably) the mesenteric vessels of tetraodontids, two infect the hearts of rachycentrids and one the heart of a mullid.

One further species requires consideration; *Aporocotyle odhneri* (Layman, 1930) was described from the "blood of the intestinal wall" of *Takifugu porphyreus* Temminck & Schlegel from off east Russia [50]. McIntosh [51] reassigned this species to *Paradeontacylix* on the basis of the arrangement and location of the testes, ovary and uterus. Given that the seven other species of *Paradeontacylix* infect the branchial and cardiac vessels of kingfish (Perciformes: Carangidae), we regard this classification as unlikely. Aside from infection site, *P. odhneri* resembles some species of *Psettarium* in aspects of anatomy including a lobed, post-testicular ovary, the posterior position of the oötype and the position of the genital pores, but it appears to lack a postlateral protuberance, which occurs in all tetraodontid-infecting species of *Psettarium*. We suspect that the affinities of *P. odhneri* are with those of other tetraodontid-infecting species, rather than with *Paradeontacylix*. The brevity of the original description, highly stylised nature of the accompanying illustrations and our inability to examine the single known specimen, however, mean that we are not in a position to propose recombination of this species.

4.3 New concept of Psettarium

In the present study, we found that the three new species formed a strongly supported clade with *Psettarium sinense*, sister to *Psettarium nolani* n. comb. We conclude that, despite the dramatic morphological variation encompassed by these species, especially in the form of the testis/testes, these species are all best considered representatives of a single genus, for which *Psettarium* is the oldest available name. Although there are no sequence data available for the type-species, *P. japonicum*, its morphology and likely infection of the body cavity of a tetraodontid forms a convincing link between it and the taxa for which sequence data are available. In this conception of the genus, *Psettarium* now comprises nine species, of which six infect tetraodontids (with the possibility of a seventh), two infect a rachycentrid and one a mullid. We think it possible, perhaps likely, that the non-tetraodontid-infecting species will prove to be unrelated to those from tetraodontids when they are analysed with sequence data, but for the present we accept that *Psettarium* remains the best classification for these taxa. Our findings mean that some aspects of the diagnosis of *Psettarium* proposed by Bullard & Overstreet [46] require amendment. We thus propose an emended diagnosis for the genus (see Results section 3.1).

Bullard & Overstreet [46] diagnosed *Psettarium* as having species in which the oesophagus is ≤25% of total body length; the oesophageal length of *P. jimbaranensis* n. sp., *P. ogawai* n. sp. and *P. pulchellum* n. sp. are 30.2%, 30.8% and 37.2% respectively. Bullard & Overstreet also diagnosed the posterior caeca as being ≥7 × longer than the anterior caeca; the posterior caeca of *P. jimbaranensis* n. sp., *P. ogawai* n. sp. and *P. pulchellum* n. sp. are, respectively, only 3.8 ×, 4.4 × and 3.2 × length of anterior caeca. It should also be noted that the posterior caeca of *P. sinense* are 5.7 × the length of the anterior caeca, while those of *P. nolani* n. comb. are only 1.4 × the length of the anterior caeca, based on measurements in the original descriptions [20, 52]. We therefore regard neither oesophageal length nor relative length of posterior caeca to anterior caeca as useful for diagnosing *Psettarium*.

The three new species, along with *Psettarium nolani* n. comb., differ from the descriptions of the other known species of *Psettarium* in having an oral sucker. The distribution of oral suckers in aporocotylids is apparently patchy, even between taxa of the same genus; for example, an oral sucker has been described for 18 species of *Cardicola*, but is absent or not reported for 9 (not including those species considered *incertae sedis* [22]). The oral sucker of many aporocotylids has been described as frail or weakly-developed (e.g. [22, 53]), and may be missed in poorly-fixed samples, hence requiring caution in interpretation [54]. While it is possible that the oral sucker has been overlooked for some species, others certainly lack it [55] or may lose it in transition from juvenile to adult stage [56]. The issue can only be resolved by re-examination of specimens of the species concerned. For the present, we have added the possible presence of an oral sucker to the genus diagnosis.

An important feature of the new *Psettarium* species reported here is what we have referred to as a degenerate posterior testis. A posterior testis, i.e. one occurring posterior to the genital pore and in addition to an anterior testis or testes, has been reported for species of six aporocotylid genera: *Acipensericola* Bullard, Snyder, Jensen & Overstreet, 2008, *Adelomyllos* Nolan & Cribb, 2004, *Chaulioleptos* Nolan & Cribb, 2005, *Neoparacardicola* Yamaguti, 1970, *Paracardicola* and *Phthinomita* [57-61]. Nolan & Cribb [59], however, note that even when spermatogenetic tissue was present, the posterior testis in species of *Phthinomita* varied dramatically even within a single species and was often apparently degenerate, containing no spermatogenetic tissue or sperm. A number of aporocotylids, e.g. species of *Adelomyllos*, *Neoparacardicola* and *Paracardicola*, have a large anterior testis and a much smaller posterior one; we see this as consistent with a pattern (potentially implemented more than once) of reduction of the posterior testis to the point where it may be non-functional as a testis, but functional as a seminal reservoir or where it may disappear entirely (or be present, but difficult to detect). The term ‘auxiliary seminal vesicle’ has been invoked for structures with no spermatogenetic function and that perhaps serve instead as storage receptacles for excess sperm; they have only been noted for species of two other aporocotylid genera: *Cruoricola* Herbert, Shaharom-Harrison & Overstreet, 1994 and *Pearsonellum* Overstreet & Køie, 1986 [62, 63]. We conclude that auxiliary seminal vesicles in aporocotylids are better interpreted, and henceforth should be referred to, as degenerate posterior testes. In our opinion, this terminology gives a superior indication of their apparent homology. The variability in size and volume of posterior testes may affect detectability [62]; however, it appears that, like oral suckers, they may not be consistent features across an entire genus, with some species (e.g. *Pearsonellum lemusi* Bullard, 2012) lacking them entirely [64]. The genus *Psettarium* has hence been rediagnosed to allow for the presence of a degenerate posterior testis in its species. This change means that the genus *Sasala*, differentiated from *Psettarium* chiefly on the basis of the presence of a posterior testis, shares diagnostic features with the latter. We have thus proposed recombination of the sole species of *Sasala*, *S. nolani*, with *Psettarium*.

The most striking variation among the new species is in the morphology of the anterior testis or testes. Most species of *Psettarium* have a single large testis between the posterior caeca. Of the three new species, *P. jimbaranensis* n. sp. possesses such a testis. However, *P. ogawai* n. sp. has multiple testes arranged mostly post-caecally and *P. pulchellum* n. sp. has a single small, globular, entirely post-caecal testis. Testis morphology and number is thus highly plastic among species of *Psettarium*. This finding has precedent in the Trematoda. Species of *Siphomutabilus* Miller & Cribb, 2013 (Cryptogonimidae Ward, 1917), possess either two or nine testes; molecular sequencing demonstrated the relatedness of the species

involved, despite this morphological disparity [65]. Similarly, schistosomatids previously classified as species of *Orientobilharzia* Dutt & Srivastava, 1955 on the basis of the possession of multiple testes have been shown to be nested within *Schistosoma* Weinland, 1858, on the basis of molecular analysis [66, 67]. The reasons behind the plasticity of testes in trematodes are unknown, but presumably relate to sexual selection, theorised as the primary driving mechanism behind the rapid evolution of male genitalia in other organisms [68-71]. It is clear from our findings that the testis is a highly plastic anatomical feature and one of the quickest to evolve in this group of parasites. We therefore regard testis number and anatomy to be uninformative for diagnosing *Psettarium*. Unfortunately, this conclusion has implications for the development of keys to the Aporocotylidae, as number and position of testes has previously been an obvious and simple character to use in genus-level distinction [72].

A final note of interest regarding the issue of testis morphology concerns species of the genus *Littorellicola* Bullard, 2010. The genus was proposed by Bullard [54] to accommodate a species of heart-infecting aporocotylid from the Florida pompano, *Trachinotus carolinus* Linnaeus (Carangidae), and a second species, *L. sebastodorum* (Holmes, 1971), originally described by Holmes [73] as *Psettarium sebastodorum*, from the hearts of rockfishes (Sebastidae, genus *Sebastes*) of the American Pacific coast. Unlike all then-previously-described *Psettarium* species, *P. sebastodorum* does not infect a tetraodontid. Holmes [73] had described *P. sebastodorum* as having a single testis. Bullard [54], however, inspected specimens of *P. sebastodorum*, concluded that it possesses multiple testes and incorporated it into *Littorellicola*. We note, however, that the existence of species of *Psettarium* with multiple testes means that the distinction between *Littorellicola* and *Psettarium* is no longer clear-cut. It will be of great interest to obtain molecular data for *Littorellicola* spp., to explore their relationships with species of *Psettarium*.

The core finding to emerge from our molecular analysis is that there has been rapid morphological diversification of the aporocotylids infecting tetraodontid fishes, evidenced by the short branch lengths between members of the clade. This phenomenon is in contrast to other aporocotylid groups, for which molecular analysis shows higher levels of molecular divergence, despite retention of morphological similarity. Thus, multiple species of *Aporocotyle, Cardicola* and *Phthinomita* (ITS2 only for the latter) exhibit higher molecular divergence than that observed for species of *Psettarium*, but relatively little morphological variation. *Paradeontacylix* is the only aporocotylid genus that exhibits a comparable level of molecular divergence to that seen among species of *Psettarium*. This genus has diversified in carangid fishes, including a complex of species infecting greater amberjack, *Seriola dumerili* Risso, in both the Mediterranean (*P. balearicus* & *P. ibericus*) and off Japan (*P.*

grandispinus & *P. kampachi*) [74, 75]. With as little as 0.2% base-pair difference between Mediterranean and Japanese species, the morphology between species is also highly conserved. By comparison, two *Psettarium* species of equivalent molecular divergence, for example *P. jimbaranensis* n. sp. and *P. ogawai* n. sp., differ dramatically in testicular morphology. The disparities between species of *Psettarium* illustrate the speed at which certain anatomical features can diverge among what are clearly closely-related species. This in turn has implications for our understanding of aporocotylid systematics. While several studies have characterised the genetic interrelationships of aporocotylid taxa in depth, e.g. Nolan & Cribb [59] and Orélis-Ribeiro *et al*. [49], many aporocotylid genera have not been genetically characterised. It is therefore possible that further examples of rapid morphological radiation may be found through analysis of known aporocotylid species.

4.4 Recognition of new species

All three new species possess features characteristic of the genus *Psettarium*, i.e. a lanceolate body shape with sinistro-lateral posterior protuberance, marginal transverse rows of minute straight spines, X-shaped caeca with posterior caeca much longer than anterior caeca, cirrus-sac, post-testicular ovary, oötype posterior to uterus and oviduct and vitelline duct between uterus and dextral body margin. We are therefore confident that all three belong to the genus *Psettarium*, as is further suggested by molecular phylogenetic analyses. All three species, however, differ from the descriptions of all of their congeners (except *P. nolani* n. comb.) in having an oral sucker and a degenerate posterior testis; they are readily differentiated from one another by the morphology of their anterior testes. *Psettarium ogawai* n. sp. possesses multiple anterior testes numbering between 16 and 22, arranged medially in an alternating ‘cobblestone’ pattern, whereas *Psettarium pulchellum* n. sp. has a small, post-caecal spheroid testis. *Psettarium jimbaranensis* n. sp. possesses a large medial testis with irregular, slightly lobed margins, extending anteriorly to the commissure of anterior and posterior caeca, posteriorly beyond the extremities of the posterior caeca and laterally beyond the caecal margins. In this regard, *P. jimbaranensis* n. sp. resembles the other species of *Psettarium* which infect tetraodontids as well as the cobia-infecting *P. rachycentri*, though it differs from all of their current descriptions in possessing a degenerate posterior testis and an oral sucker. It differs from *P. sinense* in not having a testis with deep bilateral lobes or an irregularly quadrate-shaped ovary. *Psettarium jimbaranensis* n. sp. is distinguished from *P. japonicum* by its more slender body and longer, more sinuous oesophagus that broadens posteriorly instead of remaining uniform in breadth. It differs from *P. tropicum* in having longer anterior caeca (8.5% of body length, as opposed to 3.2%) and

an ovary not divided into two fan-shaped lobes. Finally, *Psettarium jimbaranensis* n. sp. is differentiated from *P. rachycentri* in having subequal posterior caeca that do not have dendritic lateral projections and do not reach the level of the ovary, a dextral rather than medial ovary that is not deeply lobed, and a female genital pore that is sinistrally (not medially) directed.

The description of these new species brings the total of aporocotylid species described from the Tetraodontidae to 10 (see Table 4), and there are now six species known from pufferfishes of the genus *Arothron*. There are 15 recognised species of *Arothron*, with the core distribution of this genus being the Indo-Pacific region and only two species extending into the southeastern Atlantic [76]. Our discoveries bring the number of known aporocotylid hosts in this genus to four, equal to that for *Takifugu* Abe. The discovery of *Psettarium pulchellum* n. sp. in *Tylerius spinosissimus* is the first record of an aporocotylid in this genus. There is no phylogenetic information on the relationship between *T. spinosissimus* and other tetraodontids; however it is known that species of *Takifugu*, *Sphoeroides* and *Arothron*, all of which host aporocotylids, are only distantly related to one another [77]. Little is known regarding what determines host distribution and specificity for aporocotylids and, so far, no instances of infection have been reported in any other tetraodontid genera. It is clear that there remains significant potential for further aporocotylid richness to be discovered from this family of fishes.

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common two-banded seabream *Diplodus vulgaris* (Geoffrey Saint-Hilaire, 1817) (Teleostei: Sparidae): Description, molecular phylogeny, habitat and pathology. Parasitol Int. 2011;60:34–44.
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List of figures

Fig. 1: Three new species of Aporocotylidae from Balinese pufferfishes: A. *Psettarium jimbaranensis* n. sp. Holotype, dorsal view. B. *P. jimbaranensis* n. sp. Holotype, dorsal view, showing extent of vitelline follicles with those covering caeca and gonads excluded. C. *Psettarium ogawai* n. sp. Holotype, ventral view. D. *Psettarium ogawai* n. sp. Holotype, ventral view, with vitelline follicles excluded to show caeca. E. *Psettarium pulchellum* n. sp. Holotype, dorsal view. Scale-bars 300 µm.

Fig. 2: Terminal genitalia of three new species of Aporocotylidae from Balinese pufferfishes: A. *Psettarium jimbaranensis* n. sp. Holotype, dorsal view. B. *Psettarium ogawai* n. sp. Paratype, ventral view. C. *Psettarium pulchellum* n. sp. Paratype, dorsal view. Paths of vitelline ducts shown in dotted line so as to not obscure oviduct. Abbreviations: CS- cirrus sac; E- egg; FGP- female genital pore; Od- oviduct; Oo- öotype; Ov- ovary; PT- posterior testis; SV- seminal vesicle; Tt- testis; Ut- uterus. Scale-bars: A-B, 100 µm; C, 150 µm.

Fig. 3: Photomicrograph of the oral sucker of *Psettarium ogawai* n. sp., showing paired terminal papillae. Scale-bar 50 µm.

Fig. 4: Photomicrograph of *Psettarium ogawai* n. sp., showing arrangement of the multiple testes (Tt). Scale-bar 20 µm.

Fig. 5: Phylogram based on Bayesian inference and Maximum Likelihood analyses of the partial 28S rDNA region for members of the Aporocotylidae, including three new species of Balinese pufferfishes. Posterior probabilities are shown above the nodes and bootstrap support values below, with values of <50 not shown. Species infecting fishes of the family Tetraodontidae are demarcated in grey.

Figure 1

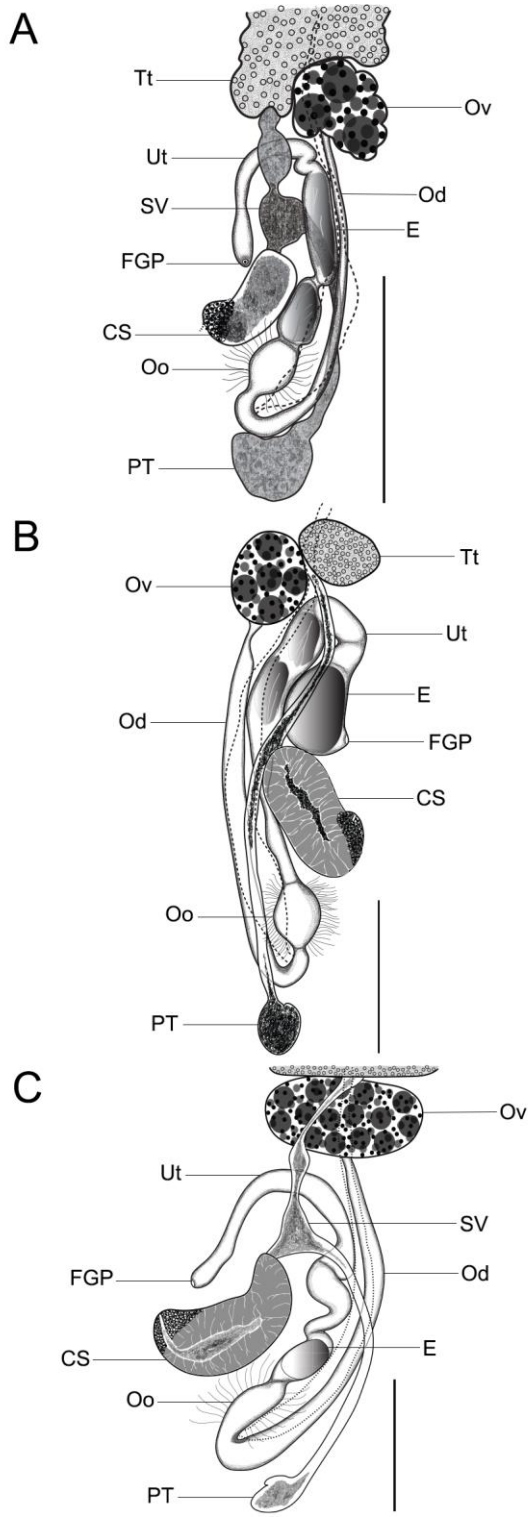

Figure 2

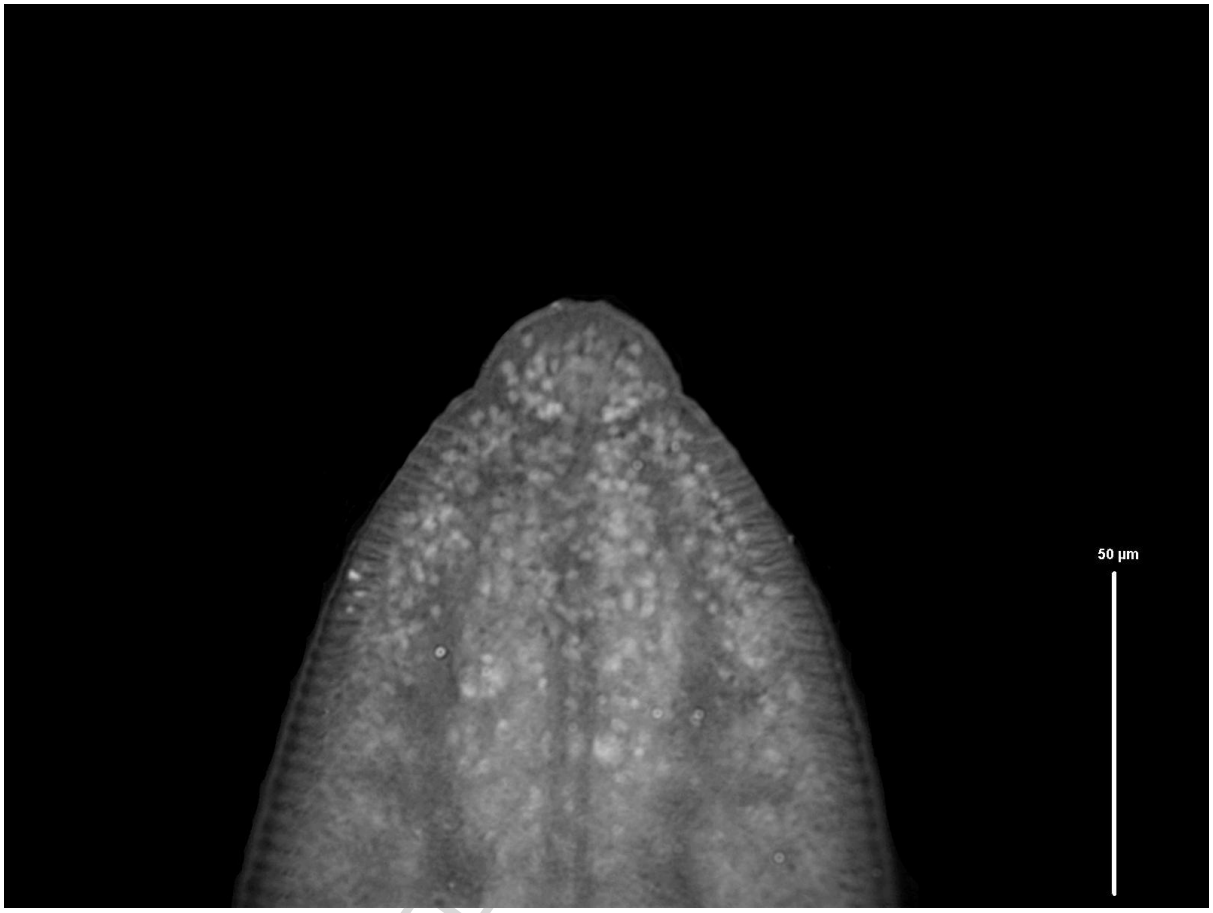

Figure 3

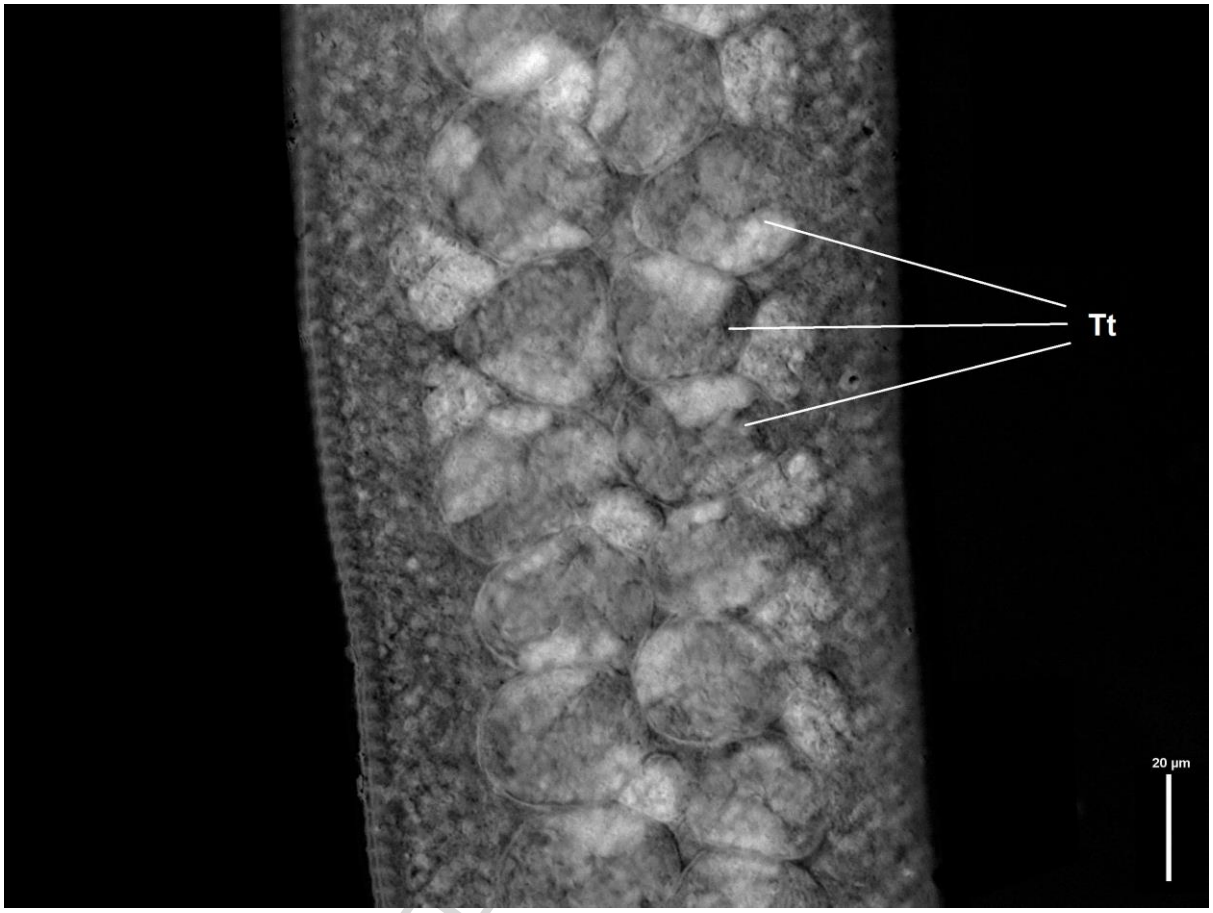

Figure 4

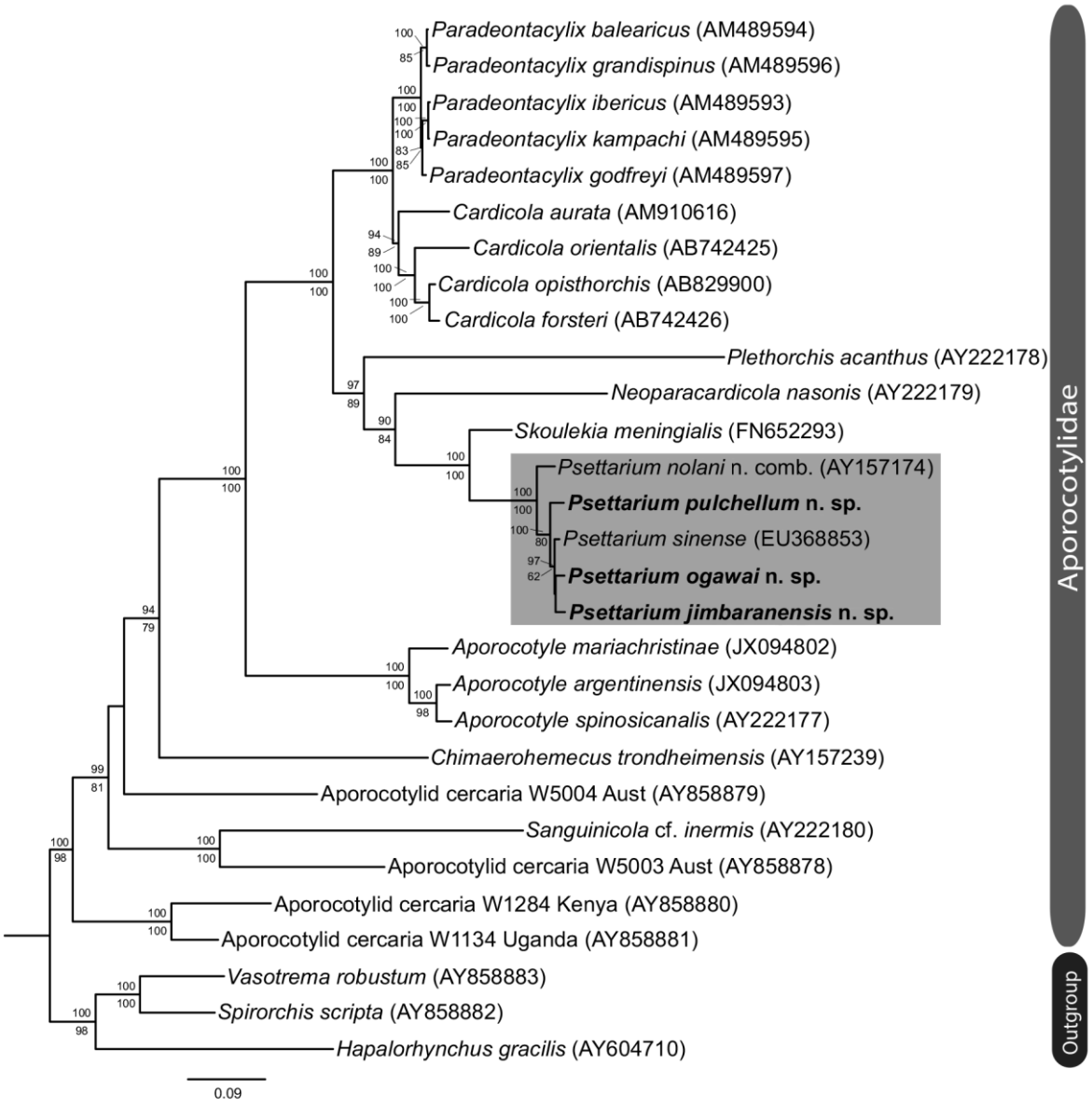

Figure 5

Table 1: GenBank accession numbers for ITS2 and partial 28S rDNA sequences generated during this study. The number of sequence replicates are in parenthesis.

Species	GenBank accession no.	
	28S rDNA	ITS2 rDNA
Psettarium jimbaranensis n. sp.	XXXXXXXX (1)	XXXXXXXX (1)
Psettarium nolani n. comb.		XXXXXXXX (3)
Psettarium ogawai n. sp.	XXXXXXXX (2)	XXXXXXXX (2)
Psettarium pulchellum n. sp.	XXXXXXXX (4)	XXXXXXXX (4)

Table 2: Partial 28S rDNA sequences from GenBank included in this study. Hosts marked with a symbol are intermediate annelid (*) or gastropod (†) hosts, from which cercariae were obtained and sequenced.

Species	Host species	GenBank accession no.	References
Family Aporocotylidae			
Aporocotylid cercaria "W1134 Uganda"	*Biomphalaria sudanica*†	AY858881	[79]
Aporocotylid cercaria "W1284 Kenya"	*Segmentorbis kanisaensis*†	AY858880	[79]
Aporocotylid cercaria "W5003 Australia"	*Thiara balannensis*†	AY858878	[79]
Aporocotylid cercaria "W5004 Australia"	*Glyptophysa gibbosa*†	AY858879	[79]
Aporocotyle argentinensis	*Merluccius hubbsi*	JX094803	[16]
Aporocotyle mariachristinae	*Genypterus blacodes*	JX094802	[16]
Aporocotyle spinosicanalis	*Merluccius merluccius*	AY222177	[80]
Cardicola aurata	*Sparus aurata*	AM910616	[14]
Cardicola forsteri	*Thunnus maccoyii*	AB742426	[81]
Cardicola opisthorchis	*Terebella* sp.*	AB829900	[18]
Cardicola orientalis	*Thunnus maccoyii*	AB742425	[81]
Chimaerohemecus trondheimensis	*Chimaera monstrosa*	AY157239	[80]
Neoparacardicola nasonis	*Naso unicornis*	AY222179	[80]
Paradeontacylix balearicus	*Seriola dumerili*	AM489594	[75]
Paradeontacylix godfreyi	*Seriola lalandi*	AM489597	[75]
Paradeontacylix grandispinus	*Seriola dumerili*	AM489596	[75]
Paradeontacylix ibericus	*Seriola dumerili*	AM489593	[75]
Paradeontacylix kampachi	*Seriola dumerili*	AM489595	[75]
Plethorchis acanthus	*Mugil cephalus*	AY222178	[80]
Psettarium nolani n. comb. (as *Sasala nolani*)	*Arothron meleagris*	AY157174	[82]
Psettarium sinense (as *Paradeontacylix sinensis*)	*Takifugu rubripes*	EU368853	
Sanguinicola cf. *inermis*	*Lymnaea stagnalis*†	AY222180	[80]
Skoulekia meningialis	*Diplodus vulgaris*	FN652293	[83]
Family Spirorchiidae			
Hapalorhynchus gracilis	*Chelydra serpentina*	AY604710	[84]
Spirorchis scripta	*Chrysemys picta*	AY858882	[79]
Vasotrema robustum	*Apalone spinifera*	AY858883	[79]
Excluded sequences			
Cardicola coeptus	*Siganus vulpinus*	JF803977	[17]
Cardicola currani	*Sciaenops ocellatus*	KJ572524	[49]

Cardicola euzeti	*Lutjanus campechanus*	KJ572526	[49]
Cardicola palmeri	*Pogonias cromis*	KJ572525	[49]
Paracardicoloides yamagutii	*Anguilla reinhardtii*	PYU42562	[85]

Table 3: Total pairwise differences among sequences of tetraodontid-infecting aporocotylids, with ITS2 sequences above and 28S sequences below the diagonal. Replicate sequences of *Psettarium ogawai* n. sp. and *P. pulchella* n. sp., showing a single base-pair difference in the ITS2 region, are provided.

Species	1	2	3	4	5	6	7	8
1. *Psettarium jimbaranensis* n. sp.	-	8	9	10	12	13	26	75
2. *Psettarium sinense*	12	-	10	11	10	11	25	71
3. *Psettarium ogawai* n. sp.	15	12	-	1	15	16	26	72
4. *Psettarium ogawai* n. sp.	15	12	0	-	14	15	25	73
5. *Psettarium pulchellum* n. sp. ex *T. spinosissimus*	23	19	21	21	-	1	26	75
6. *Psettarium pulchellum* n. sp. ex *A. manilensis*	23	19	21	21	0	-	27	76
7. *Psettarium nolani* n. comb.	38	32	40	40	40	40	-	77
8. *Skoulekia meningialis*	98	95	92	92	96	96	91	-

Table 4: List of aporocotylid species known from the Tetraodontidae, their hosts and type-localities.

Species	Host	Locality	References
Paracardicola hawaiensis Martin, 1960	*Arothron hispidus*	Hawaii, USA	[78]
Paradeontacylix odhneri (Layman, 1930)	*Takifugu porphyreus*	Peter the Great Bay, Russia	[50], [51]
Psettarium japonicum (Goto & Ozaki, 1929)	*Takifugu pardalis* *Takifugu rubripes*	Sea of Japan, Japan	[39-41]
Psettarium jimbaranensis n. sp.	*Arothron reticularis*	Bali, Indonesia	Current study
Psettarium nolani (Bray, Cribb & Littlewood, 2012) n. comb.	*Arothron meleagris*	Moorea, French Polynesia	[20]
Psettarium ogawai n. sp.	*Arothron reticularis*	Bali, Indonesia	Current study
Psettarium pulchellum n. sp.	*Arothron manilensis* *Tylerius spinosissimus*	Bali, Indonesia	Current study
Psettarium sinense (Liu, 1997)	*Takifugu oblongus* *Takifugu rubripes*	Fujian Province, China Japan	[47], [48]
Psettarium tropicum Manter, 1940	*Sphoeroides annulatus*	San Francisco, Ecuador	[43]
Rhaphidotrema kiatkiongi Yong & Cribb, 2011	*Arothron hispidus*	Lizard Island, Australia	[19]

Graphical abstract

Highlights

– Three new species of Aporocotylidae infecting pufferfishes from Bali are described

– The number of aporocotylids known from pufferfishes of the genus *Arothron* is doubled

– Molecular analysis indicates the presence of a clade of aporocotylids in pufferfishes

– All known aporocotylids in this clade are inferred to be of the genus *Psettarium*

– This study is part of the first systematic parasitological survey of fishes from Bali